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PRINCIPAL INVESTIGATOR: Junjie Chen, Ph.D.

CONTRACTING ORGANIZATION: Brigham and Women's Hospital
Boston, Massachusetts 02115

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6. AUTHOR(S)
Junjie Chen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Brigham and Women's Hospital
Boston, Massachusetts 02115

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Tumor suppressor protein p53 interacts directly with the DNA replication factor RPA and inhibits its ability to bind single-strand DNA. We defined the domain of p53 that bound to RPA and constructed p53 mutants that failed to bind RPA, but still functioned as transcriptional activators. We found that while these mutants of p53 lost their ability to bind RPA, they still maintained the growth suppression function of p53. Growth suppression function of p53 is dependent on its transactivation activity, probably by inducing p21 and other cell cycle inhibitors. We have extended our study to the p21 protein, which is induced by p53 and interacts with both the cdk2 kinase and a DNA replication factor PCNA. Here we have demonstrated the importance of PCNA-inhibitory domain of p21 in vivo. We have also shown that p21 has to interact directly with both cyclin subunit and cdk2 subunit of the cyclin-cdk complex in order to inhibit the kinase activity and suppress cell growth in vivo.

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ANNUAL REPORT (DAMA17-94-J-4070)

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The following work is done under the supervise of Dr. Anindya Dutta, who is currently supported by a Career Development grant (DAMD17-94-J-4064). I am a postdoctoral fellow in Dr. Anindya Dutta's lab. Our independent grants only support our own salaries. There is some overlap in the work described below because it is being done in concert by postdoctoral fellow and advisor.

The first part of the work is focused on the interaction of tumor suppressor p53 and Replication Protein A (RPA), based on the earlier finding by Dr. Dutta that p53 can interact with RPA and inhibit its function. Our interests in the function of p53 in the development of breast cancer led us to investigate an important cell cycle regulatory protein p21, which is the major downstream regulatory protein of p53. p21 can not only inhibit cyclin/cdk kinase activity, but also interact directly with and inhibit an essential DNA replication factor, proliferating cell nuclear antigen (PCNA). Last year we reported that the region of p21 involved in interacting with and inhibiting cyclin/cdk complexes could be separated from the region that interacted with and inhibited PCNA. Using these separated domains we showed that cyclin-cdk2 inhibitory domain of p21 is primarily responsible for the growth suppression activity of p21, while PCNA-binding domain of p21 can inhibit the SV40 based DNA replication reaction. Here we report our further studies on p21 as the second part of this report.

INTRODUCTION

Cancer can be characterized as a deregulation of normal cellular proliferation controls. The connection between cell cycle controls and cancer is well established. Several tumor suppressor genes which are functionally inactivated in human cancers, such as p53 and Rb (Retinoblastoma protein) exert their primary effect at the G1-S transition in the cell cycle. Tumor Suppressor p53 has been the major focus of the cancer research, because it is mutated in more than 50% of human cancers including breast cancer (1). More and more evidence suggest the essential role of p53 in cancer development. Deletion of the p53 gene in "knock-out" mice confers a very high risk of cancer. Several viral oncogenes, including E6 gene of human papilloma virus and large T antigen of Simian virus 40, can specifically inactivate p53 by various mechanisms. p53 has multiple biological functions: overexpression of p53 can arrest cell cycle at G1 to S transition; p53 is essential for DNA repair after mild DNA damage; p53 is also required for apoptosis following extensive DNA damage. The transforming mutants of p53 are defective in all these functions.

The exact mechanisms by which p53 carry out these biological functions are still unclear p53 is a transcriptional activator that has been shown to activate a number of cellular genes including cellular oncogene mdm2, DNA repair gene GADD45 and cell cycle regulatory p21 gene. p53 can also bind to TATA-box binding protein TBP, and suppress general transcription from promoters lacking p53 binding sites. Both transcription activation and suppression activity of p53 can contribute to its various biological functions. Recently, more biochemical activities of p53 have been identified: p53 can bind to insertion-deletion mismatch lesions (2); p53 is also found to have 3' to 5' exonuclease activity (3). Three years ago, our group (4) found that p53 could directly bind to DNA Replication Protein A (RPA) and inhibit its activity. This finding suggested a novel mechanism of p53 function, that is inhibiting DNA replication by directly interacting with RPA.

To test whether the ability to interact with RPA is important for the replication inhibitory property of p53, we mapped the interaction domains of RPA and p53. By direct mutagenesis, we obtained mutations of p53 that failed to bind RPA but still function as a transcription activator. We also obtained several p53 mutants from Dr. A. Levine that were transcriptional inactive but still bound RPA. Here, we tested whether the binding to RPA is important for the growth suppression function of p53.

The transcriptional activity of p53 results in the increased expression of several human genes, one of which is the cell-cycle regulator, p21. Transcription of p21 is induced by p53 upon DNA damage (5)."knock-out" experiment show that p21 is required for cell cycle arrest upon DNA damage (6). Overexpression of p21 can arrest cells at G1-S transition just like p53 (5, 7, 8). All these data suggest that p21 protein is a major effector of the growth suppression function

of p53. Because of the importance of p21 as a major down-stream effector of p53, we have begun studying the mechanisms by which p21 arrest cell cycle.

The transition from G1 to S phase of the cell cycle is controlled by the activation of several cyclin/cdks. p21/CIP1 can inhibit all cyclin/cdk complexes. p21 has also been shown to interact directly with and inhibit an essential DNA replication factor, proliferating cell nuclear antigen (PCNA) (9, 10, 11, 12). Recently, we showed that the region of p21 (N-terminal half of p21: p21N) involved in interacting with and inhibiting cyclin/cdk complexes could be separated from the region (C-terminal half of p21: p21C) that interacted with and inhibited PCNA (12). Using these separated domains we showed that p21N inhibits DNA replication in Xenopus egg extracts and inhibits growth of a transformed human osteosarcoma cell line (SaOs2), while p21C inhibits the SV40 based DNA replication reaction. These results suggest that the primary growth suppression property of p21 is due to its inhibition of cyclin/cdk complexes (12).

The ability of p21N alone to inhibit cell-cycle progression has interesting implications for the activity of other cdk inhibitors like p27 (up-regulated by TGFb, (13, 14)) and p57 (15, 16) which show homology with p21 in the region contained in p21N. In order to understand the mechanism by which p21 inhibit the cyclin-cdk2 complexes, we further narrowed down the cdk2 inhibitive domains. Here, we report that p21 can bind directly to both cyclins that cdk2. This finding is particularly interesting, because p27-cyclin A-cdk2 crystal structure was published last month (17) and confirmed that p27/p21 interacted with both cyclin and cdk2. Our studies further demonstrate that both cyclin-binding domain and cdk2-binding domain are important for the kinase inhibitory activity and growth suppression activity of p21.

Although inhibition of PCNA by p21 (and p21C) is not apparently required in the growth suppression assay. One explanation is that transformed cells may have an excess of PCNA, so that higher levels of p21C are required to inhibit PCNA function. Further mapping suggests that C-terminal 38 amino acids are necessary and sufficient for the binding of PCNA. Here, we used a more sensitive assay (microinjection) to test whether PCNA binding activity of p21 can inhibit DNA replication *in vivo*.

BODY

SPECIFIC AIMS FOR YEAR 2

1. By mapping the interaction domains of RPA p70 and p53, we made several mutations in p53 which abolish its binding to RPA p70. We analyzed in detail the effect of these mutations on the transcriptional suppression and growth suppression activities of p53 (Year 1, Task 2).

2. Examine whether the p21-PCNA interaction contributes to growth suppression by p21(new).

3. Studying the mechanism by which p21 inhibit cyclin-cdk2 complexes (new).

METHODS

Expression Plasmids and Baculoviruses.

Plasmids utilized for expression of various proteins in bacteria were pETp21His (11), pETp27His (13), pETCdk2 (12), pGST-RbC (18), pGST-cyclin D1,2,3 (Dr. Yue Xiong), pGST-cyclin E (19). A BamH I-Hind III fragment of cyclin E from pGST-cyclin E was cloned into the E. coli expression vector pRSET (Invitrogen) to generate pRSET-cyclin E (26-402).

Plasmids containing the mutant alleles of p21 were obtained from Dr. J. Smith (20). PCR with N- and C- terminal oligonucleotides was used to clone these mutant alleles of p21 into pGEX-5X-3. pGEX-p21D1-29 was made by restriction enzyme digestion of a plasmid expressing GST-p21 using a Pvu II site in the coding region of p21. pGEXp21D17-24 was made by PCR based strategy using appropriately designed oligonucleotides. Fragments containing p21 coding regions with deletions at either amino acids 17-24 or amino acids 53-58 also were cloned into pETHis (13) for expression of His-tagged p21 derivatives in bacteria.

pCMVCdk2, pCMVCdk4, pRcCyclin A, pRcCyclin B, pRcCyclin E, pRcCyclin D1, D2, D3 was kindly provided by Dr. P. Hinds, and used for the expression of cyclins and Cdks by *in vitro* transcription and translation.

Baculoviruses expressing Cdk2, GST-cyclin A, GST-cyclin B and GST-cyclin E were obtained from Dr. Helen Piwnica-Worms. Baculoviruses expressing cyclin D1, 2, 3 were gifts from Dr. C. J. Sherr. Baculoviruses expressing GST-Cdk4 was provided by Dr. J. W. Harper (see (21) and references therein).

Mammalian cell expression constructs containing full length p21 and p21N have been described previously (12). p21(D17-24) and p21N (D17-24) were cloned into pcDNA3 (Invitrogen) using the same strategy. pCMV/p53 mutants 14-19, 22-23, 48-49 and 61-62 were the kind gift of Dr. Arnold Levine. p53 wild type and W53S-F54S mutants were cloned into a mammalian expression vector cDNA3 (Invitrogen).

Protein expression and purification.

Bacterial expression of proteins were performed in E. coli strain BL21. Protein induction, cell lysis and affinity-purification with glutathione-agarose beads (Sigma) were done as described (4).

Hi-5 cells were infected with recombinant baculoviruses containing various cyclins and cdks as described (21). Active cyclin/Cdk complexes were affinity-purified with glutathione-agarose beads (Sigma).

Protein expression by *in vitro* transcription and translation were performed using TnT coupled Rabbit Reticulocyte Lysate system (Promega).

Peptides (PS100-PS103) were synthesized by Research Genetics Inc. PS100: ACRRLFGPVDSE; PS101: ACRRLKKPVDSE; PS102: FYHSKRRLIFSK; PS103: FYHSKRDDIFSK. A 41 amino acid p21C2 peptide (consisting of the 39 C-terminal amino acids of p21 and two lysine residues at the carboxy-terminal end required for chemical synthesis) was synthesized at the Harvard Medical School Biopolymer Laboratory.

p21C2: QAEGSPGGPGDSQGRKRRQTSMTDFYHSKRRLIFSKRKPKK
BWH262: WNSGFESYGSSSYGGAGGYTQAPGGFGAPAPSQAEKKSRAR (control peptide from N terminus of human RPA p34).

Pull down assay, immunoprecipitation and immunoblotting.

Pull down assays were performed as described (12). Basically, 100-300 ng of GST fusion protein and 5-10 µl of bacterial or reticulocyte lysate in 200 µl of buffer A7.4 (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.01% NP40, 10% glycerol, 25 mM NaCl) were incubated one hour at 4 degrees on a rotating wheel. Proteins associated with GST fusion proteins were pulled down with glutathione agarose beads. After washing the beads 4 times in binding buffer, bound proteins were eluted by boiling in Laemmli's SDS-PAGE sample buffer for 10 minutes, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and detected either by autoradiography (for radiolabeled proteins expressed by in vitro transcription and translation reactions using rabbit reticulocyte lysate) or by western blotting using appropriate antibodies. In each experiment, care was taken to equalize the amount of input proteins and GST protein was included as a negative control. For peptide competition, the indicated peptides were included in the reactions at a concentration of 75 µM. Conditions used for the immunoprecipitation-immunoblot assay with human cell lysates were as described (22).

The antibodies used in this study were monoclonal antibodies to cyclin E (HE12, HE172) and polyclonal antibodies to p27 (J. Massague), cdk2 (Santa Cruz), cdk4 (H. Chou), cyclin A (J. Pines), and cyclin D1 (L. Zuckerman). The monoclonal antibodies (CP2, CP36, CP68) to p21 were generated against full length recombinant human p21 (22). CP2, CP36, CP68 recognize the amino acids 1-17, 17-24 and 130-150 of p21, respectively. This was determined by immunoblotting a panel of bacterially produced deletion derivatives of p21.

Microinjection.

IMR90 human diploid fibroblast monolayers growing on glass coverslips (at 60% density) were synchronized in G0 by serum starvation for 48 hr. and stimulated to enter G1 by addition of 10% fetal bovine serum. 15 hr after re-activation, cells in G1 were microinjected with the indicated proteins using an automated microinjection system (AIS, Zeiss). All microinjection experiments were carried out in 3.5 cm Petri dishes containing 3 ml of carbonate-free DMEM, in order to avoid a decrease in pH of the medium during the injection. Each cell was injected with protein or peptide (3.75 mg/ml in PBS) together with normal rabbit immunoglobulin (2.5 mg/ml) at a pressure between 50 and 150 hPa. The computer settings for injection were: angle "45", speed "10" and time of injection "0.0 seconds", so as to deliver 0.01 to 0.05 pL of liquid per nucleus. For more details on the microinjection procedure, see (23).

DNA synthesis was monitored by incubating with BrdU (100 µM, Amersham) for 10-12 hr after microinjection. Coverslips were then rinsed in PBS and fixed for 10 min in -20°C cold methanol-acetone (1:1) and washed again three times with PBS. Micro-injected cells were detected by incubation for one hour with biotinylated horse anti-rabbit IgG (Vector Laboratories, dilution 1:50), washed three times with PBS and incubated with Texas red-conjugated streptavidin (Vector Laboratories, dilution 1:100). Coverslips were subsequently incubated for 10 min with 1.5 M HCl, washed three times with PBS and then incubated for one hour with a solution of mouse monoclonal anti-BrdU antibody plus nuclease (undiluted, Amersham), followed by a 30 minutes incubation with 1:50 dilution of an anti-mouse FITC-conjugated antibody (Vector Laboratories).

All antibody reactions were carried out in a humidified chamber at room-temperature and dilutions were made in DMEM containing 10% FCS. Counterstaining for DNA was performed by adding 1 µg/ml bisbenzimide (Hoechst 33258) into the final PBS wash. Immunofluorescence samples were directly mounted in Crystal/mount medium (Biomeda Corp.). Photographs were taken using a Plan-Neofluar 40 X lens mounted on a Zeiss Axiophot Photomicroscope and a Color Video Printer Mavigraph, on Sony UPC-3010 print paper.

In each experiment about 100 injected cells (and corresponding number of non-injected cells) were counted. % of Inhibition of BrdU incorporation was calculated as {(N-I)/N} x 100, where N is the percentage of BrdU incorporation in non-injected cells and I is the percentage of BrdU in cells microinjected with antibodies. The obtained numerical value is independent of possible experimental variations in the number of BrdU positive cells that had not been injected.

Kinase assay, SV40 and Xenopus DNA replication assays.

Kinase assays were performed for 15 min at 30°C using 1 ng of insect cell-expressed cyclin/Cdk complexes and 3 μg of purified GST-RbC (C-terminal portion of Retinoblastoma protein Rb) in 25 ml of kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT and 50 μM ATP containing 5 μCi [g-^{32}P] ATP) with other indicated components. Kinase reactions were stopped by the addition of 2X Laemmli sample buffer and then boiled for 10 minutes. The products were analyzed by SDS-PAGE. Phosphorylation of the substrate was quantitated both by liquid scintillation counting of bands excised from gels and by phosphorimager analysis. Ki_{app} is the concentration of inhibitor at which kinase activity is inhibited by 50%.

SV40 and Xenopus DNA replication assays were carried out as described (12).

Transcription repression assay.

SaOs2 or H1299 cells were transfected with 10 ug of plasmids expressing p53 alleles (based on the cDNA3 vector), 5 ug of a reporter plasmid expressing the beta-galactosidase gene from a cytomegalovirus. Eight hours later, plates were washed twice in PBS and fresh medium (DMEM with 10% FCS) was added. After 36 hours, cells were harvested and lysed. Equal fractions of cell lysates from each of the transfected plates were assayed beta-galactosidase (transcription repression) activity (24). Beta-galactosidase activity were expressed as percentage of activity relative to plates with cDNA3 vector alone (100%).

Growth suppression assay by stable transfections.

Plasmids expressing alleles of p53 or p21 were transfected into SaOs2, a human osteosarcoma cell line with loss of both alleles of p53, as well as H1299, a human lung large cell carcinoma cell line with partial homozygous deletion of the p53 gene, by the calcium phosphate method. Exponentially growing cultures were transfected with 10 ug of each plasmid. After twenty-four hours, cells were washed in phosphate buffered saline, and fresh DMEM medium containing 10% fetal calf serum and G418 was added. The ability of each plasmid to produce G418 resistant colonies was measured as described (12).

RESULTS

Studies on the interaction of p53 and RPA:

RPA-p53 interaction is not required for the transcriptional suppression activities of p53.

Last year, we constructed two p53 mutants that did not bind RPA but still maintain their transcriptional activation activity. As p53 can also function as a transcriptional suppressor on promoters lacking p53 binding sites, we tested the effects of these mutations on transcriptional suppression activity of p53. Plasmids expressing wild type or mutant p53 were transfected into SaOs2 and H1299 cells (deficient in endogenous p53) and transcriptional suppression activity of p53 were assayed on the immediate early promoter of cytomegalovirus (CMV) using beta-galactosidase as a report gene (Figure 1A). The two mutants p53 that did not bind RPA can suppress transcription from the CMV promoter as well as wild type, suggesting that RPA-p53 interaction is not required for transcriptional suppression activity of p53.

RPA-p53 interaction is not required for the growth suppression activities of p53.

To examine the growth suppression activity of p53, plasmids expressing wild type or mutant p53 were transfected into SaOs2 and H1299 cells (deficient in endogenous p53) and G418 resistant colonies were selected (Figure 1B). Plasmids expressing wild type p53 established very few G418 resistant colonies compared to the vector, due to growth suppression by p53. Two mutants of p53 (D48H-D49H and W53S-F54S) inhibited cell growth as well as wild type p53, indicating that RPA-p53 interaction is not required for the growth suppression activity of p53.

The p53 protein with the mutation of amino acids L22Q-W23S, which had wild type RPA binding activity but reduced transcription activation and repression activities, showed diminished growth suppression in both SaOs2 and H1299 cells. The L14Q-F19S and D61H-E62K mutants, which retained most of the transcriptional activation and repression functions, also retained most of the growth suppression activity of wild-type p53 in both SaOs2 and H1299 cells. These results imply the trans-activation and/or repression properties of p53 are important for growth suppression.

Studies on p21, a downstream effector of p53:

p21 (WAF1, CIP1 or sdi1) is a protein induced by the tumor suppressor protein p53, which interacts with and inhibits two different targets essential for cell-cycle progression . One of these is the cyclin-cdk family of kinases and the other is the essential DNA replication factor PCNA. Last year, we separated domains of p21 responsible for interacting with and inhibiting these two targets and tested their function *in vivo*. While cdk-inhibitory domain could inhibit cell cycle on its own, PCNA-inhibitory domain of p21 could not inhibit cell cycle in the growth suppression assay we used. Here we used a more sensitive assay, microinjection, to examine whether PCNA-inhibitory domain of p21 can arrest cell cycle in vivo. Furthermore, we studied the mechanism by which p21 inhibits cyclin-cdk kinase activity.

Inhibition of the SV40 replication reaction by GST-p21C2 and p21C2 peptide.

In the first year of this study, we defined a 39 amino acid fragment of p21 (p21C2) which is necessary and sufficient to bind PCNA. Since the interaction of p21 with PCNA inactivates its function as a DNA replication factor, we measured the abilities of the GST fusion proteins to inhibit the SV40 based DNA replication reaction (Figure 2). The concentration required to obtain 50% inhibition of replication (IC50) was 0.5 to 1 nM for GST-p21 or GST-p21C and 9

nM for GST-p21C2. The synthetic p21C2 peptide was slightly weaker than GST-p21C2 at inhibiting SV40 replication (IC50 = 14 nM). The 10-20 fold weaker inhibitory activity of GST-p21C2 compared to GST-p21C is consistent with its lower affinity for PCNA at 37°C. The inhibition of DNA replication by p21C2 was reversed by the addition of excess PCNA (data not shown). GST-p21M1, unable to bind PCNA (data not shown), did not inhibit the DNA replication reaction (Figure 2). These results suggest that the C-terminal 39 amino acids of p21 is an effective inhibitor of DNA replication in vitro, although weaker compared to GST-p21 or GST-p21C.

C-terminal part of p21 can prevent quiescent cells entry into S phase.

To determine whether C-terminal of p21 can interact with an inhibit PCNA in vivo, we analyzed whether S phase was inhibited by these proteins. Quiescent diploid fibroblasts were stimulated by serum and entry into S phase followed after micro-injection of GST-fusion proteins or the p21C2 peptide (Figure 3). GST-p21 -p21N and -p21C inhibited uptake of Bromodeoxyuridine significantly compared to a negative control peptide CSH119, GST alone, or GST fused to a cell-cycle regulatory protein cdc25C. Thus, GST-p21C inhibits growth of cells almost as well as GST-p21N when provided in high enough concentrations. Consistent with the result from the in vitro SV40 replication reaction, GST-p21C2 inhibited entry into S phase although less effectively than GST-p21C. The p21C2 peptide was only a weak inhibitor of cell growth. Nevertheless, these results not only confirm earlier reports that p21N, which binds and inhibits cdk kinases but not PCNA, inhibits cell growth, but also suggest that p21C, which binds and inhibits PCNA, can also inhibits cell growth if provided at high concentration.

Cyclin E associates stably with p21 or p27 independent of the catalytic cdk2 subunit.

As we showed previously, the primary growth suppression property of p21 is due to its ability to inhibit cyclin-cdk complexes. p21 has been found to bind directly to the cdk2 subunit of the cyclin-cdk complex. Bacterially expressed p21 or p27 was mixed with glutathione agarose beads containing equal amounts of GST-cyclin fusion proteins to test the direct association of p21 or p27 with various cyclins (Figure 4A, B, Fig. 6A). GST-cyclin E was best at associating with p21 and p27, although weak interaction could be seen with GST-cyclin A and B (Figure 4B). Cyclin A-p21 association is best seen in Figure 6A where the amount of p21 input is ten times more than in Figure 4A.

Cyclin E and p21 associated with each other even when both GST-p21 and cyclin E were produced in bacteria (Figure 4C, lane 3). The same was true for cyclin E and p27 (data not shown). Various deletion derivatives of p21 were generated to define which portion of p21 was responsible for the interaction with cyclin E. p21 with a deletion of amino acids 17-24 failed to bind cyclin E (Figure 4C, lanes 4 and 5). The association of cyclin A with p21 was also disrupted by the deletion of amino acids 17-24 (Figure 6A, middle panel). Thus, amino acids 17-24 of p21, ACRRLFGP, form part of the cyclin binding motif of p21 and will be referred to as the Cy1 site. This sequence is highly conserved (7/8 identity) among the cdk inhibitors p21, p27 and p57.

The same deletion derivatives of p21 were used to bind bacterially expressed cdk2 (Fig. 4D). Amino acids 53 to 58 of p21 were essential for association with cdk2 (lane 8), consistent with published studies showing that this region is required for association with and inhibition of cyclin/cdk kinases (9, 30). We have designated this cdk binding motif of p21 as the K site. Therefore, the cyclin binding Cy1 site is distinct from the cdk2 binding K site.

Taken together, cyclin E or cyclin A can stably associate with p21 or p27 independent of cdk2. The association of p21 or p27 with cyclin E appears stronger than that with cyclin A.

The Cy1 site of p21 is important for interaction with cyclin/cdk complexes.

We next tested whether the Cy1 site of p21 was important for the interaction of p21 with cyclin/cdk complexes (results summarized in Table 1). Cdk2, cdk4, cyclin E, cyclin A and cyclin D1 were produced separately in rabbit reticulocyte lysates by *in vitro* transcription-translation (Figure 5). When cyclin E or A were synthesized alone, immunoprecipitation with anti-cdk2 antibody demonstrated that the radiolabeled cyclin was associated with rabbit cdk2 from the reticulocyte lysate (Figure 5A, lane 2 and data not shown). Likewise, when cyclin D1 was produced in the rabbit lysate, it was complexed with rabbit cdk4 and cdk2 (Figure 5B, lanes 8 and 2). Cdk2 and cdk4 produced in rabbit reticulocyte lysates were free of cyclins, as none of the antibodies against cyclins immunoprecipitated the cdk subunits (data not shown).

The radiolabeled proteins were tested for association with GST-p21 and various deletion derivatives of p21. Cdk2 interacted exclusively with amino acids 53-58 of p21 (K site), because deletion of this sequence disrupted the association of cdk2 with p21 (Fig. 5C, lane 6). Cdk4 alone did not associate with p21 (Fig. 5D, lane 3). Cyclin D1/cdk4 complex associated with p21 exclusively via the Cy1 site because deletion of this site abolished all association (Fig. 5B, lanes 4 and 5; the residual cyclin D1 seen in these lanes is probably due to cyclin D1/cdk2). Unlike cdk2 alone or cyclin E alone, the association of cyclin E/cdk2 with p21 was not disrupted by mutation of either the K or the Cy1 sites (Fig. 5A, lanes 4-6), suggesting that both these sites independently interact with the cyclin E/cdk2 complex. Cyclin A/cdk2 behaved like cyclin E/cdk2 in these assays (Table 1).

To confirm the above results, GST-cyclin E, GST-cyclin E/cdk2, GST-cyclin A, GST-cyclin A/cdk2 and GST-cdk4/cyclin D1 (D2 or D3) were purified after over-expression in insect cells and tested for association with bacterially expressed p21 or deletion derivatives lacking either the Cy1 site (p21D17-24) or the K site (p21D53-58) (Fig. 6A). Deletion of the Cy1 site selectively disrupted association with cyclin A, cyclin E and cyclin D/cdk4, but did not disrupt association with cyclin A/cdk2 and cyclin E/cdk2. Deletion of the K site (D53-58) did not affect association with the individual cyclins or cyclin/cdk complexes.

To further substantiate this observation, GST-cyclin E/cdk2 complexes were mixed with bacterially produced p21 and its derivatives, and p21-associated complexes were immunoprecipitated with the anti-p21 monoclonal antibody CP68 (recognizes amino acids 130-150 of p21). Co-immunoprecipitated GST-cyclin E was detected by immunoblotting with a monoclonal antibody against cyclin E (HE12) (Fig. 6B). Consistent with the hypothesis that cyclin E/cdk2 interacts with p21 through either the Cy1 or the K sites this interaction was disrupted only when both these sites were mutated (GSTp21D17-24, D53-58).

Therefore, although discovered because of its ability to bind free cyclin E or A, the Cy1 site of p21 is used in interaction with cyclin/cdk complexes. The Cy1 site is particularly important for association with cyclin D1/cdk4. The Cy1 or K sites independently permit the association of p21 with cyclin E/cdk2.

<u>A 12 amino acid peptide containing the Cy1 site is sufficient to interact with cyclin E/cdk2 or cyclin A/cdk2</u>.

We tested whether a peptide, PS100, containing only the Cy1 site of p21 (residues 17-28, ACRRLFGPVDSE) was sufficient to interact with cyclin/cdk complexes (results summarized in Table 1). Since the cyclins produced by *in vitro* transcription and translation were associated with rabbit cdks (as shown in Fig. 5) we first tested their binding to p21D53-58 (no K site). PS100 peptide competitively inhibited the association of cyclin E/cdk2 (or cyclin A/cdk2) with p21D53-58 (Fig. 7A) while a mutant peptide (FG mutated to KK; PS101) had no effect. Therefore, a peptide with the Cy1 site alone is sufficient to interact with cyclin E/cdk2 and cyclin A/cdk2 and competitively inhibit the association of cyclin/cdks to a p21 molecule lacking a K site.

Surprisingly, the PS100 peptide also inhibited the association of cyclin E/cdk2 and cyclin A/cdk2 with wild type p21, while the mutant PS101 peptide failed to do so (identical result as Fig. 7A). This result is contradicted by the robust association of p21D17-24 with cyclin E/cdk2 (Fig. 6A, lane 5; Fig. 6B, lane 4) or cyclin A/cdk2 (Fig. 6A, lane 3). The apparent contradiction can be explained if the p21 protein has a second redundant cyclin binding site (Cy2 site).

PS100 would inhibit interaction of the cyclin/cdk complex with either the Cy1 or Cy2 sites, but deletion of only the Cy1 site in p21 would leave the Cy2-cyclin interaction un-impaired. This putative Cy2 site should be expected to have low affinity for isolated cyclin subunits, otherwise deletion of Cy1 site would not disrupt the association of p21 with isolated cyclin E or cyclin A (Fig. 4C, Fig. 6A).

Cy2 site is present in the C terminus of p21.

A second sequence (Cy2) with similarity to the Cy1 site was, in fact, identified at residues 152-158 of p21 (HSKRRLIF; the underlined sequence is most similar). To test whether Cy2 is really a cyclin binding site, PS102, a peptide containing residues 150-161 of p21, was used to compete for the association of cyclin E/cdk2 with p21D53-58. PS102 competitively inhibited the association of cyclin E/cdk2 with p21D53-58 in a manner analogous to PS100, while a mutant peptide, PS103 (RL residues changed to DD) did not (Fig. 7A, Table 1). Like PS100, PS102 also specifically disrupted the interaction of wild type p21 with cyclin E/cdk2 (data not shown).

If Cy2 is responsible for the association of p21D17-24 with cyclin E/cdk2 (Fig. 6A, lane 5; Fig. 6B, lane 4), we predict that a p21 molecule missing both the Cy1 and Cy2 sites but retaining an intact K site would fail to associate with cyclin E/cdk2. Consistent with this prediction, GSTp21C3D17-24 (containing amino acids 1-150 of p21 with the deletion of the Cy1 site) or GST-p21ND17-24 failed to associate with cyclin E/cdk2 (Fig. 6B, lane 7 and data not shown). Therefore, at least one Cy site is required for the stable interaction of p21 with cyclin/cdk complexes.

Although the failure of GST-p21D17-24 to associate with cyclin D1/cdk4 (Fig. 5B, lane 5) indicates the primary importance of the Cy1 site in the association of p21 with this complex, PS100 (Cy1 peptide) or PS102 (Cy2 peptide) were unable to block the cyclin D1/cdk4-p21 interaction (Fig. 7A and data not shown). One explanation is that the synthetic peptides were too small to contain the entire cyclin D1 interacting motif. This explanation is supported by the observation that p21C2, a longer peptide containing residues 127-164 of p21 (which includes the Cy2 site), efficiently blocked the association of cyclin D1/cdk4 with p21 (Fig. 7A). Therefore, the exact nature of the interaction of the cyclin binding motifs of p21 may be subtly different with different cyclin/cdk complexes.

The Cy regions of p21 alone weakly inhibit the kinase activity.

We tested whether peptides containing the Cy regions of p21 inhibited kinase activity. As substrate we used GST-RbC, a recombinant protein containing the C terminal portion of the retinoblastoma gene product, a physiological substrate for the G1 cyclin/cdk complexes. Peptides PS100 (Cy1 site) or PS102 (Cy2 site) inhibited cyclin E/cdk2 or cyclin A/cdk2 kinase activities (Fig. 7B; Table 2), while the mutant PS101 or PS103 peptides did not. The Cy1 peptide was a better inhibitor than the Cy2 peptide consistent with the greater avidity of the Cy1 site for the cyclins. The Cy peptides were far weaker than intact p21 or p21 with one K site and at least one Cy site intact (see text below and Table 2). However, inhibition by the wild type Cy peptides was significant since the mutant Cy peptides PS101 and PS103 did not inhibit the kinase to 50% of control even at concentrations exceeding 300 mM.

PS100 and PS102 did not inhibit cyclin D1/cdk4, as expected from the observation that they were insufficient to associate with the kinase complex. p21C2, the longer Cy2 containing peptide capable of associating with cyclin D1/cdk4, inhibited the kinase activity (Table 2).

The Cy regions of p21 are essential for kinase inhibition.

The K site of p21 is essential for kinase inhibition (Table 2; also see (30)). Because the Cy1 and Cy2 regions are important for the association of p21 with cyclin/cdk complexes (Table 1), we expect them to be similarly important for kinase inhibition. To test this, the concentration at which mutant p21 derivatives inhibited the kinase reaction to 50% of the uninhibited reaction was determined (Ki_{app}) (Table 2).

Loss of the Cy1 site of p21 or p21N abolished their abilities to inhibit cyclin D1/cdk4 kinase. These results are consistent with the absolute requirement for the Cy1 site for mediating p21- cyclin D1/cdk4 association. For cyclin A/cdk2 or cyclin E/cdk2, deletion of the Cy1 site alone (p21D17-24) increased Ki_{app} by four fold compared to wild type p21. A more dramatic effect was observed when both the Cy1 and Cy2 sites were deleted simultaneously (compare p21D17-24 with p21ND17-24 or with p21C3D17-24). These results are consistent with the observation that either the Cy1 or the Cy2 site stabilizes the interaction with cyclins E or A in the cyclin/cdk2 complexes, and double deletion of both Cy sites destabilizes the interaction (Fig. 6B, lane 7).

The Cy sites of p21 are important for suppression of Xenopus DNA replication.

If the Cy deletion derivatives of p21 lose their ability to interact with and inhibit cyclin/cdk kinases, these mutations should affect the biological activities of p21. To test this we assayed DNA replication in Xenopus egg extracts, which is sensitive to the cdk inhibitory activity of p21(17). GST-p21 or GST-p21D17-24 was titrated into the Xenopus egg extract (Fig. 8A and 8B). Loss of the Cy1 site decreased replication inhibition five-fold (IC50 of GST-p21 = 0.1 μM; IC50 of GST-p21Δ17-24 = 0.5 μM). Both proteins associated with Xenopus cdk2 and were equally stable under replication conditions (data not shown).

We also tested whether the peptide containing a Cy1 motif (PS100) was sufficient to inhibit DNA replication (Fig. 8C). PS100 peptide at a concentration of 3 mM inhibited DNA replication to 47% of the control reaction, while the mutant peptide PS101 permitted replication to 92% of the control reaction. The high concentration of PS100 required to inhibit the replication reaction is consistent with the weak inhibition of cyclin/cdk kinases by the peptide (Table 2).

The Cy sites of p21 are required for cell growth suppression.

To determine whether the Cy sites were important for the biological activity of p21 *in vivo*, we tested cell growth suppression by various deletion derivatives of p21 (3). Plasmids expressing p21 ($Cy1^+$, K^+, $Cy2^+$), p21D17-24 ($Cy1^-$, K^+, $Cy2^+$), p21N ($Cy1^+$, K^+, $Cy2^-$) and p21ND17-24 ($Cy1^-$, K^+, $Cy2^-$) were stably transfected into SaOs2 cells (p53 null, Rb null) and H1299 cells (p53 null, intact Rb) and colony formation measured (Fig. 9A). In agreement with the *in vitro* results, only p21 with neither Cy1 nor Cy2 sites (p21ND17-24) significantly lost growth suppression. Immunoblotting of transiently transfected cell extracts revealed that the steady state level of p21ND17-24 was as high as that of the growth suppressive p21 derivatives (Fig. 9B). Immunoprecipitation of the p21 derivatives followed by immunoblotting with anti-cdk2 antibody showed that only p21ND17-24 failed to associate with cdk2 (data not shown). Therefore, although p21ND17-24 associates with the isolated cdk2 subunit (Fig. 4D, lane 10 and data not shown), the interaction of the K site alone with cyclin/cdk2 kinase complex was too weak to be detected either *in vitro* (Fig. 6B) or *in vivo* (data not shown). However, addition of either Cy site stabilized the p21-cyclin/cdk interaction and inhibited cell growth.

FIGURE LEGENDS

Figure 1 A. Beta-galactosidase activity of p53 wild type and mutants transiently transfected into SaOs2 and H1299 cells. Beta-galactosidase activity represents the O.D. 420 after addition of ONPG substrate compared to vector cDNA3 alone (=100%, no transcription repression) and represents the mean ± SEM activity of 8 plates from each mutant. **B.** Growth suppression of wild type p53 and mutants in stable transfection assays. Bars represent the mean (± standard error of the mean) of the number of colonies for 14 (SaOs2) and 4-5 transfections (H1299) compared to cDNA3 (= 100%, no growth suppression). Data were analyzed by one-way ANOVA and means were categorized by Fisher's LSD test. * indicates a significant difference compared to all other p53 alleles at $p < 0.0003$ (SaOs2) and $p < 0.0001$ (H1299).

Figure 2 Inhibition of SV40 DNA replication by fragments of p21. The proteins added were GST-p21 (open squares), GST-p21C (open circles), GST-p21C2 (closed circles), GST-p21M1 (open triangles) and p21C2 peptide (closed squares). Each point represents the mean and standard deviation of three separate measurements of DNA replication (amount of dAMP incorporated into polynucleotide).

Figure 3 Inhibition of entry into S phase by microinjection of GST-p21 fusion proteins and indicated peptides into nuclei of serum re-activated diploid fibroblasts 15 hr after re-activation. Mean and standard deviation for at least three different experiments are shown. CSH119 is the negative control, with indicated growth inhibition probably being a side-effect of the injection procedure.

Figure 4 Direct interaction between p21 and p27 with cyclins. **A)** p21 associates directly with GST cyclin E. Proteins bound to indicated GST fusion proteins after incubation with an E. coli lysate containing recombinant human p21 were visualized by immunoblotting with polyclonal anti-p21 antibody. 0.1 input = one-tenth input lysate. **B)** p27 associates directly with some GST cyclins, cyclin E in particular. The lane numbers match those in part A), except that the input lysate was E. coli lysate containing $p27^{His6}$, and the protein was visualized with a polyclonal antibody to p27. **C)** Amino acids 17-24 of p21 are essential for association with cyclin E. Protein bound to GST or GST-p21 (indicated alleles) after incubation with E. coli lysate containing recombinant human cyclin E was visualized by immunoblotting with HE12 monoclonal antibody to cyclin E. The 55 kD cyclin E band is indicated. wt: wild-type p21. D1-29 : p21 with amino acids 1-29 deleted etc. **D)** Amino acids 53-58 of p21 are essential for association with cdk2, and loss of the cyclin-binding site (17-24) does not affect binding to cdk2. The lane numbers match those in C), except that the input lysate was E. coli lysate containing recombinant human cdk2, and the protein was visualized by a commercial anti-peptide antibody to cdk2. Lanes 9 and 10 contain protein bound by GST-p21N and GST-p21ND17-24.

Figure 5 Association of p21 and its derivatives with cyclin E, cyclin D1, cdk2 and cdk4. All the cyclins and cdks were produced by *in vitro* transcription and translation using rabbit reticulocyte lysate. The association of cyclins with rabbit cdks were verified by immunoprecipitation using polyclonal antibody against cdk2 (lane 2) or cdk4 (lane 8 in panel B). One-tenth input (10% of lysate used in the binding assay), radioactive translation products bound to indicated GST fusion proteins or antibodies were visualized by fluorography.

Figure 6 A) Association of cyclins or cyclin/cdk complexes with p21 and its derivatives. All cyclins and cyclin/cdk complexes were purified on glutathione agarose beads using insect cell lysates infected (or coinfected) with correspondent baculoviruses. Proteins (p21 and its derivatives) bound to indicated GST fusion proteins after incubation with E. coli lysate containing recombinant human p21, p21D17-24 or p21D53-58 were visualized by immunoblotting with polyclonal anti-p21 antibody. **B)** Double deletion of Cy1 and K sites

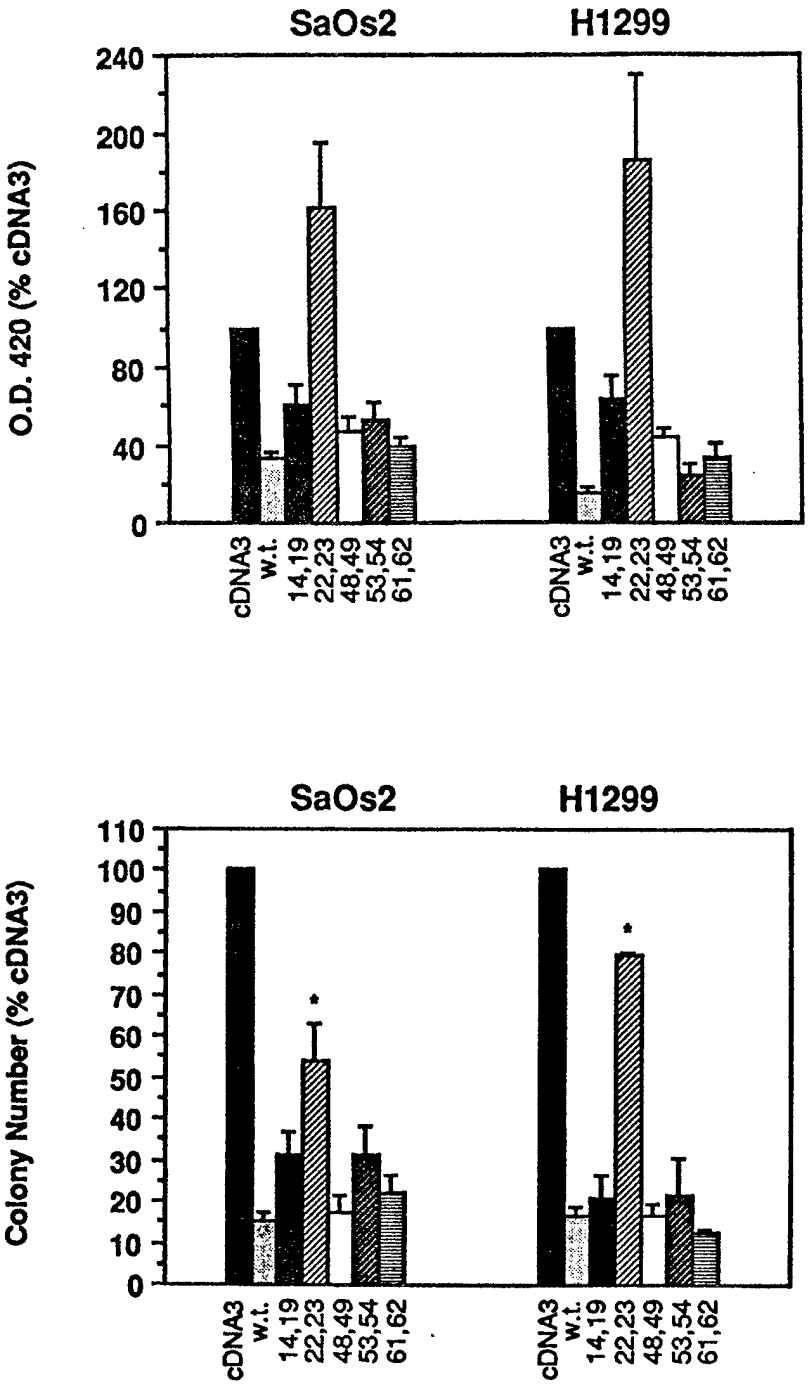
(GSTp21D17-24, D53-58) or of Cy1 and Cy2 sites (GSTp21C3D17-24) on p21 abolished its association with GST cyclin E/cdk2. Purified GST cyclin E/cdk2 protein were mixed with GST and GST p21 derivatives indicated at the top. The p21 associated proteins were immunoprecipitated with CP68 antibody (anti-p21) and immunoblotted with anti-cyclin E antibody (HE12). The bands below the GST cyclin E band were immunoglobin heavy chains (Ig h) in the immunoprecipitates.

Figure 7 **A)** Association of GST-p21D53-58 with cyclin A/cdk2 or cyclin E/cdk2 is competitively inhibited by PS100 peptide (Cy1 site) or PS102 peptide (Cy2 site), but not by mutant peptides PS101 or PS103. The same results are obtained with GST-p21. Association of cyclin D1/cdk4 with GST- p21D53-58 was not blocked by PS100 or PS102, but was blocked by a longer peptide containing the Cy2 site (p21C2). The cyclin/cdk complexes were made by *in vitro*-transcription translation as described in Table 1, and association with GST-p21D53-58 tested in the presence of 75 mM of indicated peptides. BWH262 is a negative control peptide described in the Methods. Bound radioactive cyclins were visualized by fluorography. **B)** Wild type peptide PS100 inhibits the kinase activity of cyclin E-cdk2 (E/K2) and both PS100 and PS102 inhibit the kinase activity of cyclin A-cdk2 (A/K2). The mutant peptides PS101 or PS103 do not inhibit either kinase (see Table 2 for details).

Figure 8 The Cy1 site is important for optimal inhibition of DNA replication in Xenopus egg extracts. Replication of sperm DNA in Xenopus egg extracts (with radiolabeled α^{32P} dATP) were carried out in the absence or presence of indicated proteins or peptides. In panel A, the reactions (with 1 μM of proteins indicated) were stopped at various time points, and products were visualized by autoradiography. In panels B and C, the reactions were stopped at 2 hr, the products quantitated by counting excised gel slices.

Figure 9 **A)** Inhibition of cell growth in SaOs2 cells ($p53^-$, Rb-; solid bars) and H1299 cells ($p53^-$, Rb^+; stippled bars). Number of stably transfected colonies obtained by transfection of vector alone (cDNA3) and vectors expressing indicated alleles of p21 and p21N (d= D). The results are expressed as percentage of vector control, with the mean and standard deviation of 9-12 (SaOs2) and 3-4 (H1299) independent transfections. **B)** Expression levels of the D17-24 and wild-type alleles were comparable after transient transfection of the corresponding plasmids into H1299 cells, as measured by immunoblotting transfected cell lysates (48 hours after transfection) with anti-p21 antibody.

Figure 1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O.D. 420 (% cDNA3)
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200
160
120
80
40
0
cDNA3
w.t.
14,19
22,23
48,49
53,54
61,62
SaOs2
H1299
Colony Number (% cDNA3)
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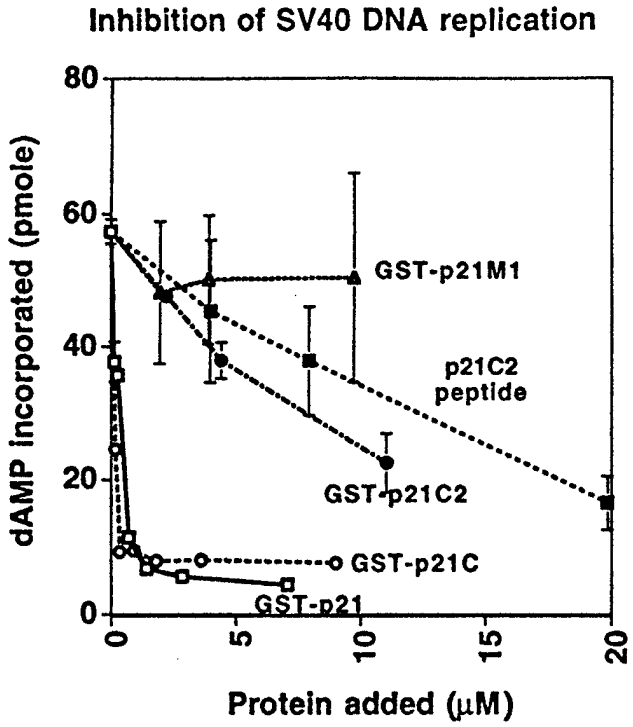
100
90
80
70
60
50
40
30
20
10
0
*
*

Figure 2


Inhibition of SV40 DNA replication
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60
40
20
0
0
5
10
15
20
Protein added (μM)
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p21C2 peptide
GST-p21C2
GST-p21C
GST-p21

Figure 3

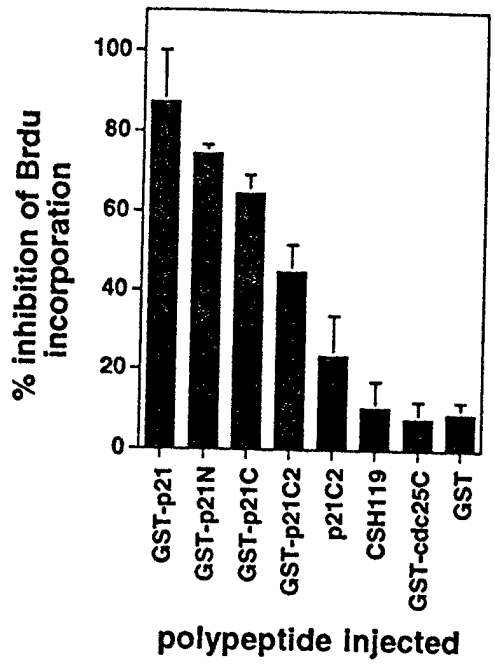
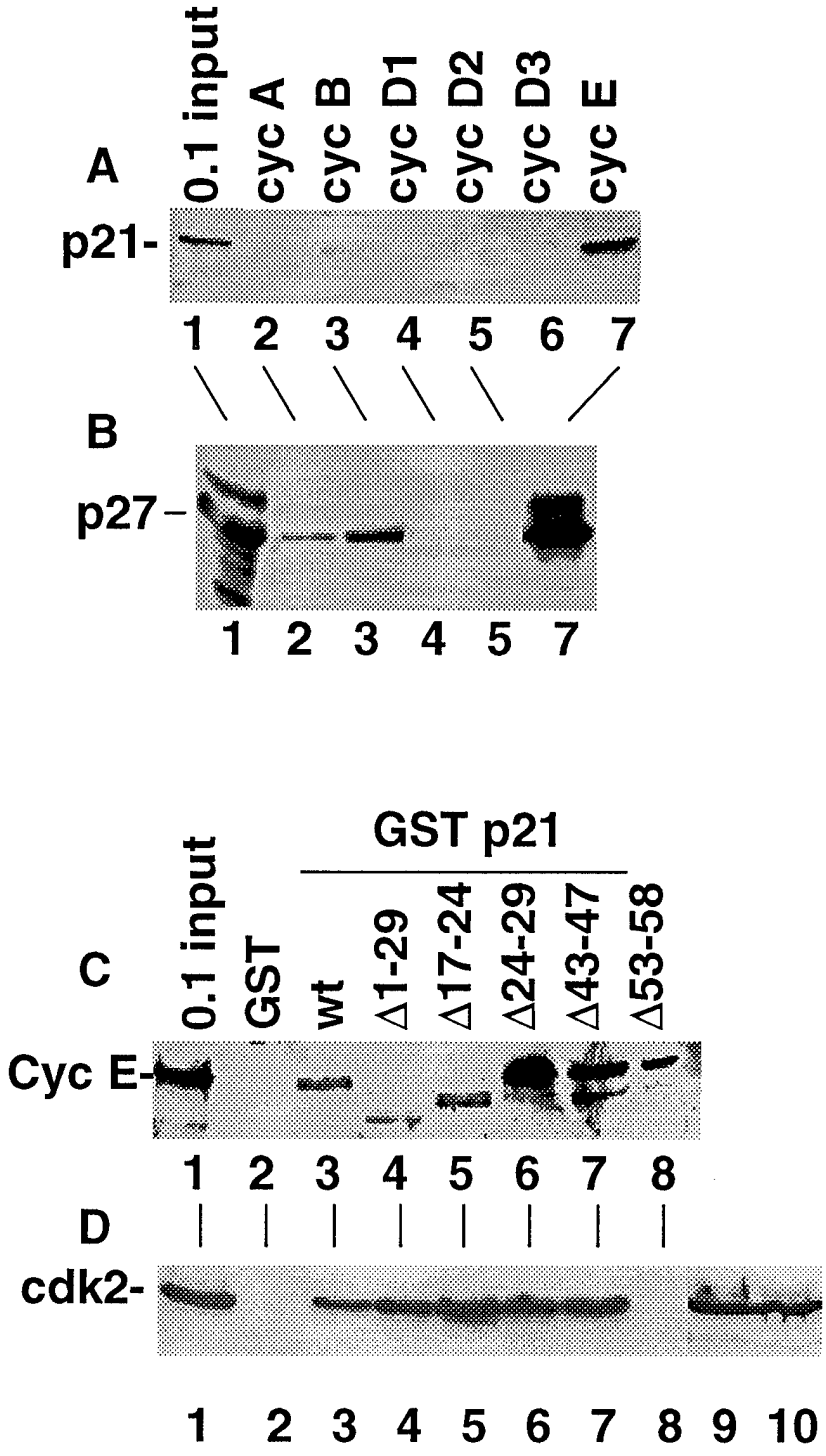

% inhibition of Brdu incorporation
100
80
60
40
20
0
GST-p21
GST-p21N
GST-p21C
GST-p21C2
p21C2
CSH119
GST-cdc25C
GST
polypeptide injected

Figure 4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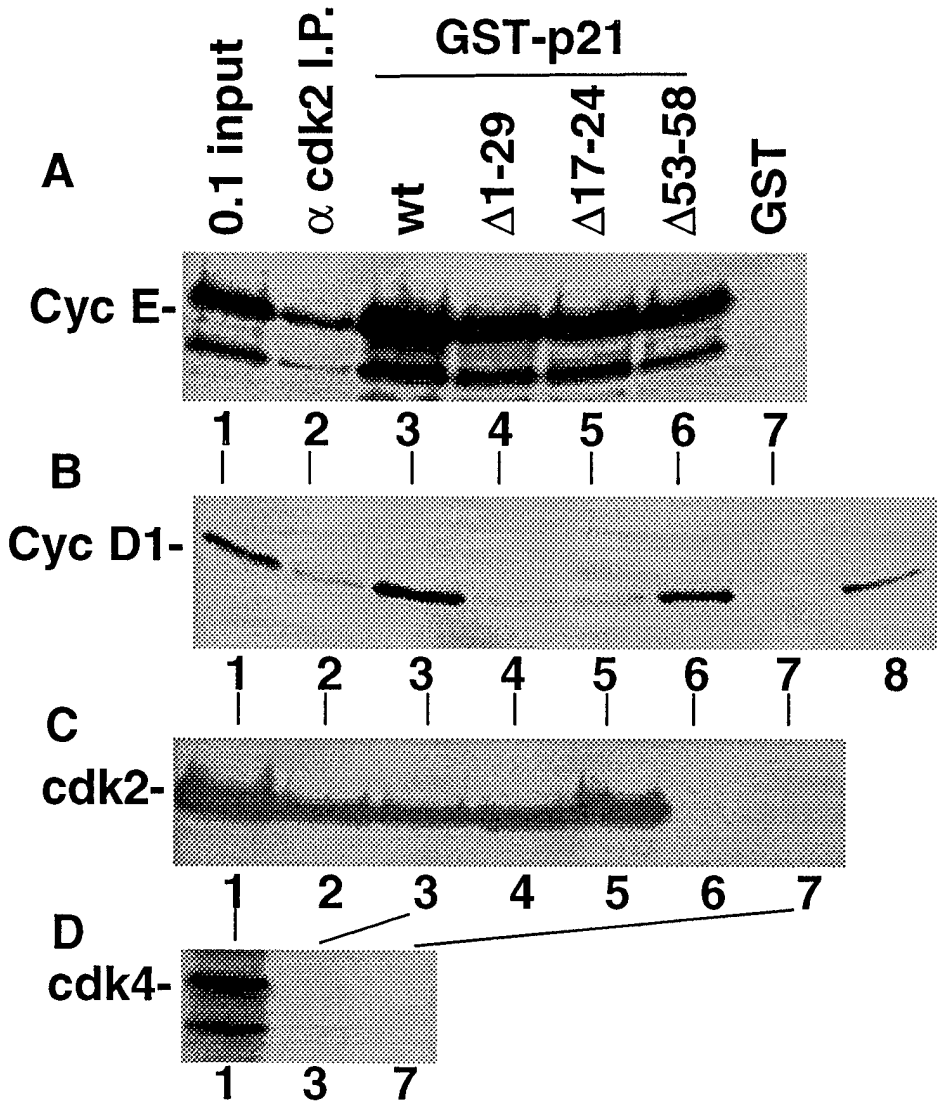
cyc A
cyc B
cyc D1
cyc D2
cyc D3
cyc E
p21-
1 2 3 4 5 6 7
B
p27-
1 2 3 4 5 7
C
GST p21
0.1 input
GST
wt
Δ1-29
Δ17-24
Δ24-29
Δ43-47
Δ53-58
Cyc E-
1 2 3 4 5 6 7 8
D
cdk2-
1 2 3 4 5 6 7 8 9 10

Figure 5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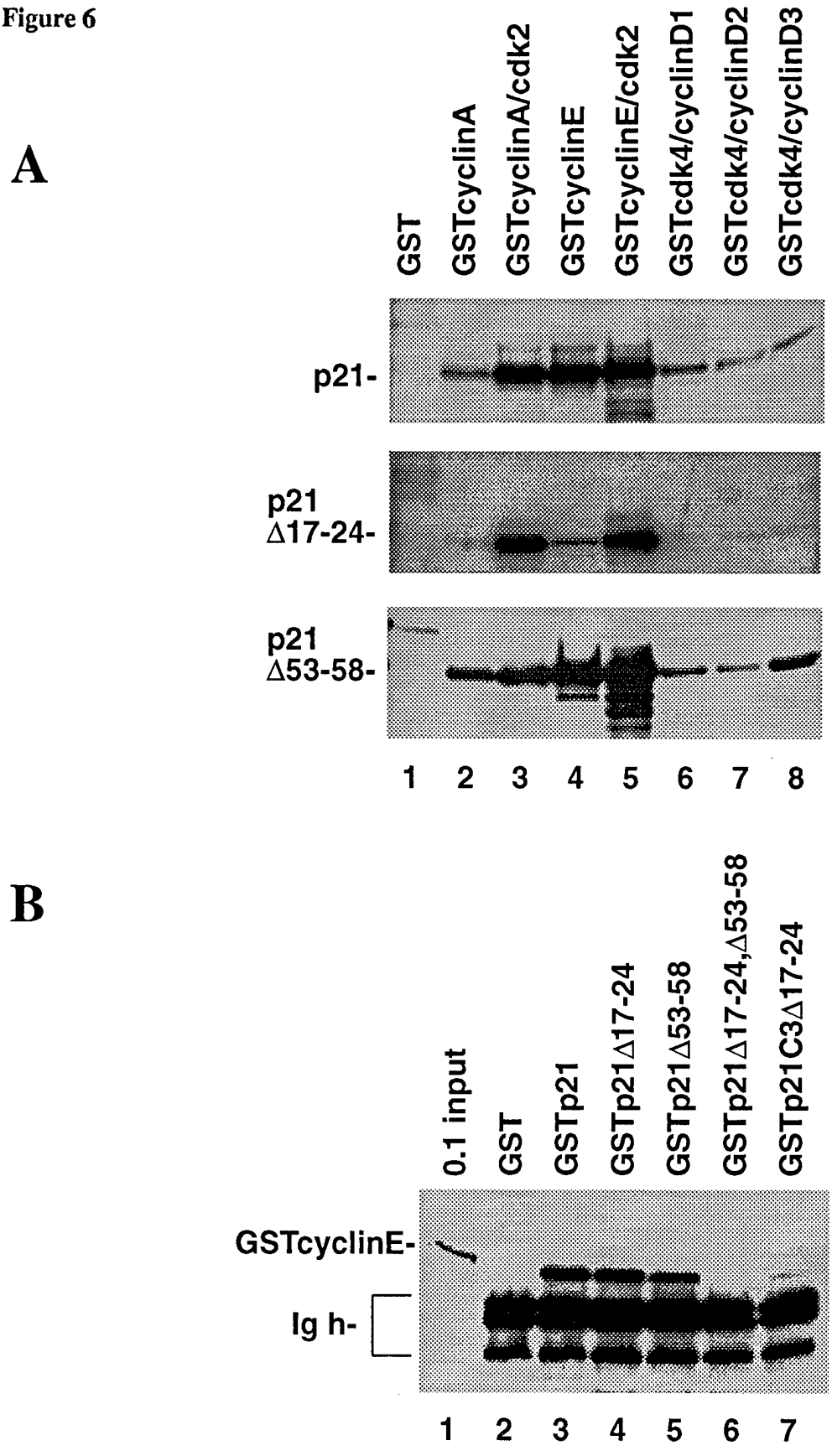
α cdk2 I.P.
GST-p21
wt
Δ1-29
Δ17-24
Δ53-58
GST
Cyc E-
1 2 3 4 5 6 7
B
Cyc D1-
1 2 3 4 5 6 7 8
C
cdk2-
1 2 3 4 5 6 7
D
cdk4-
1 3 7

Figure 6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GSTcyclinA
GSTcyclinA/cdk2
GSTcyclinE
GSTcyclinE/cdk2
GSTcdk4/cyclinD1
GSTcdk4/cyclinD2
GSTcdk4/cyclinD3
p21-
p21
Δ17-24-
p21
Δ53-58-
1 2 3 4 5 6 7 8
B
0.1 input
GST
GSTp21
GSTp21Δ17-24
GSTp21Δ53-58
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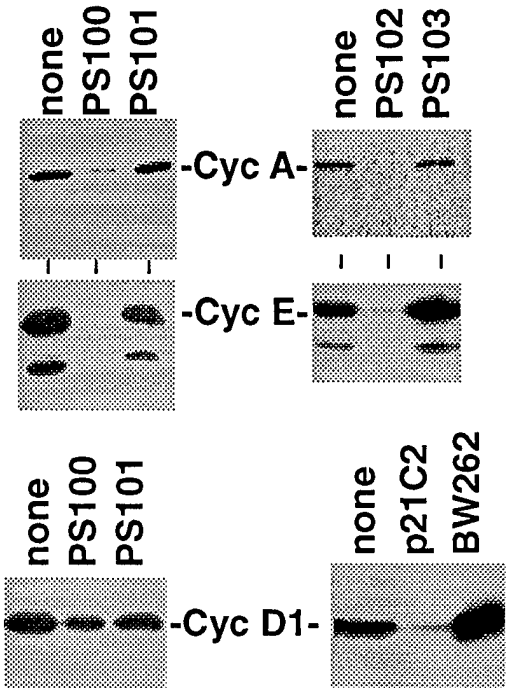
GSTp21C3Δ17-24
GSTcyclinE-
Ig h-
1 2 3 4 5 6 7

Figure 7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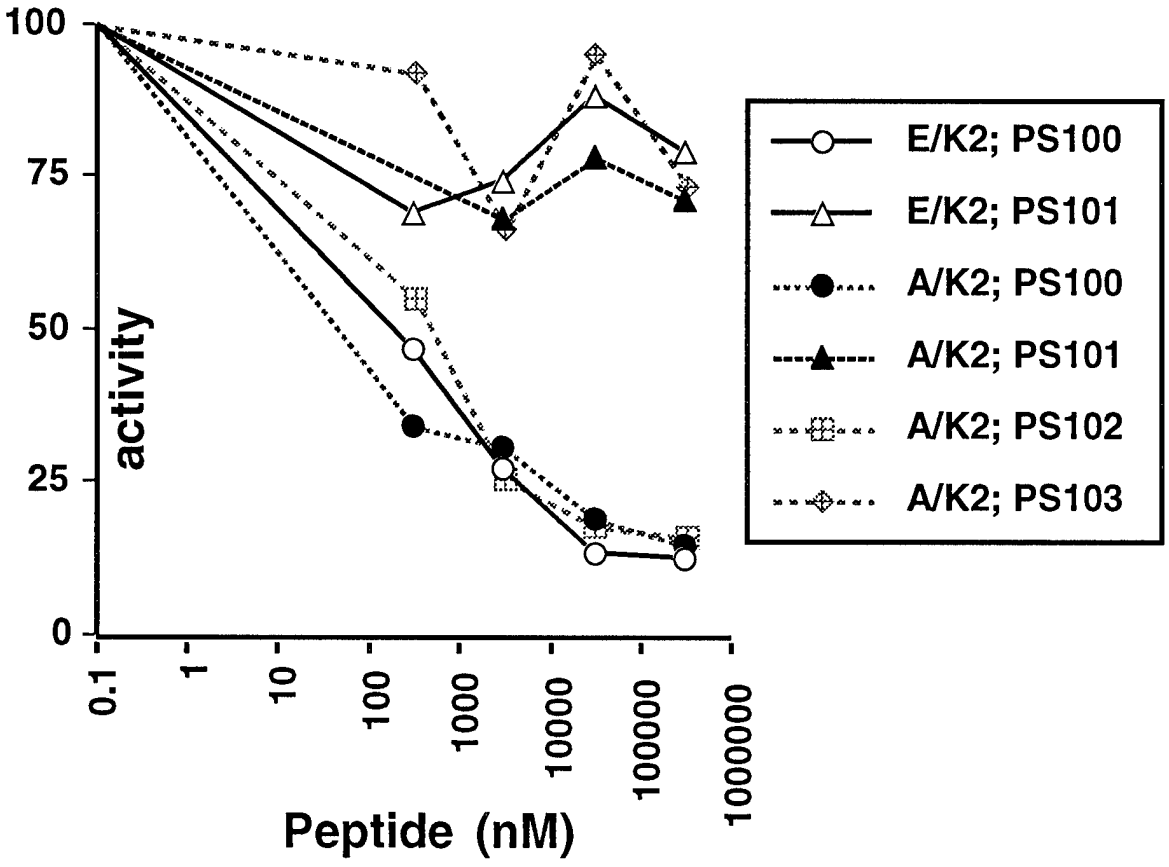
PS100
PS101
none
PS102
PS103
-Cyc A-
-Cyc E-
none
PS100
PS101
none
p21C2
BW262
-Cyc D1-


100
75
50
25
0
activity
0.1
1
10
100
1000
10000
100000
1000000
Peptide (nM)
E/K2; PS100
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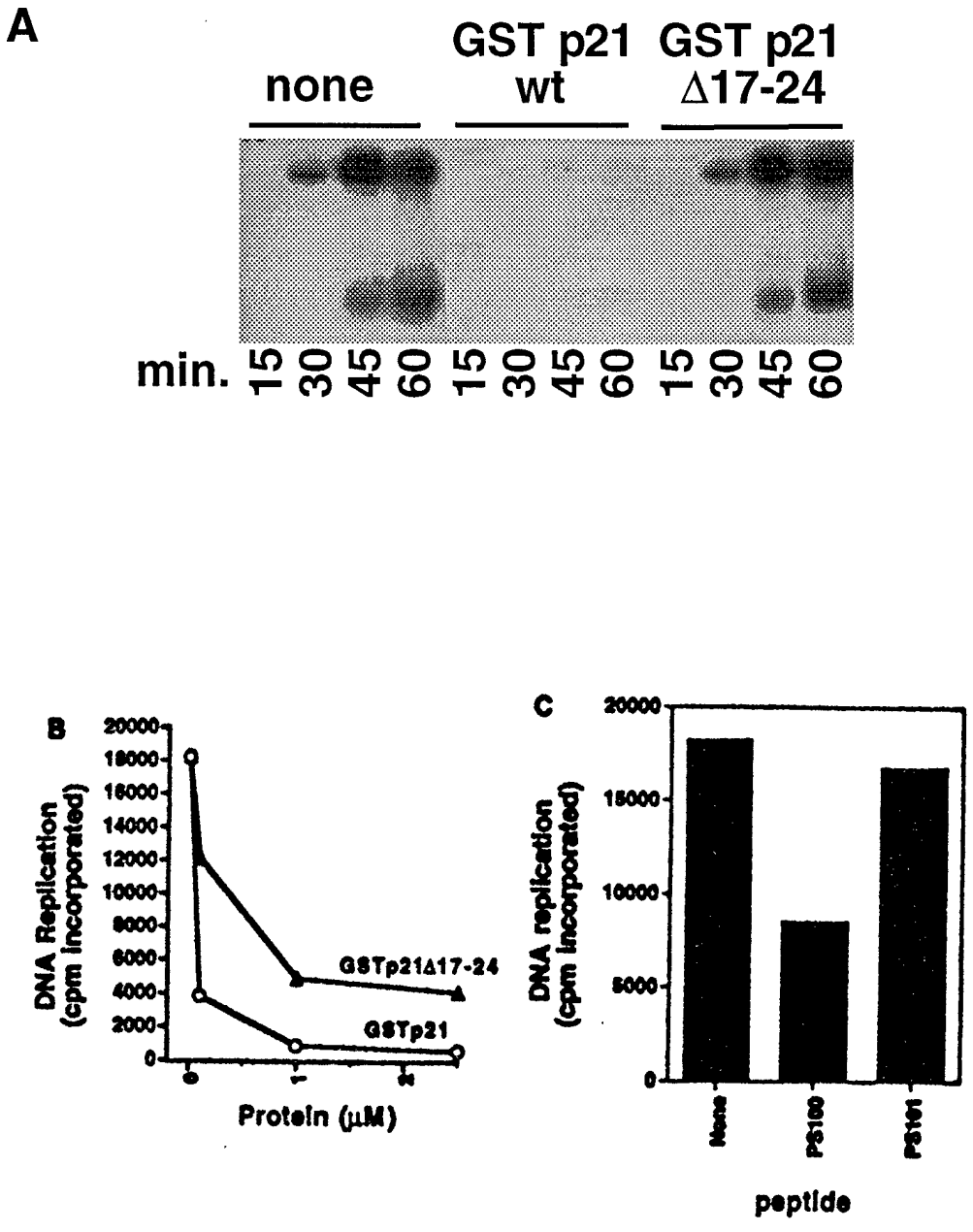
A/K2; PS100
A/K2; PS101
A/K2; PS102
A/K2; PS103

Figure 8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GST p21 wt
GST p21 Δ17-24
min. 15 30 45 60 15 30 45 60 15 30 45 60
B
DNA Replication (cpm incorporated)
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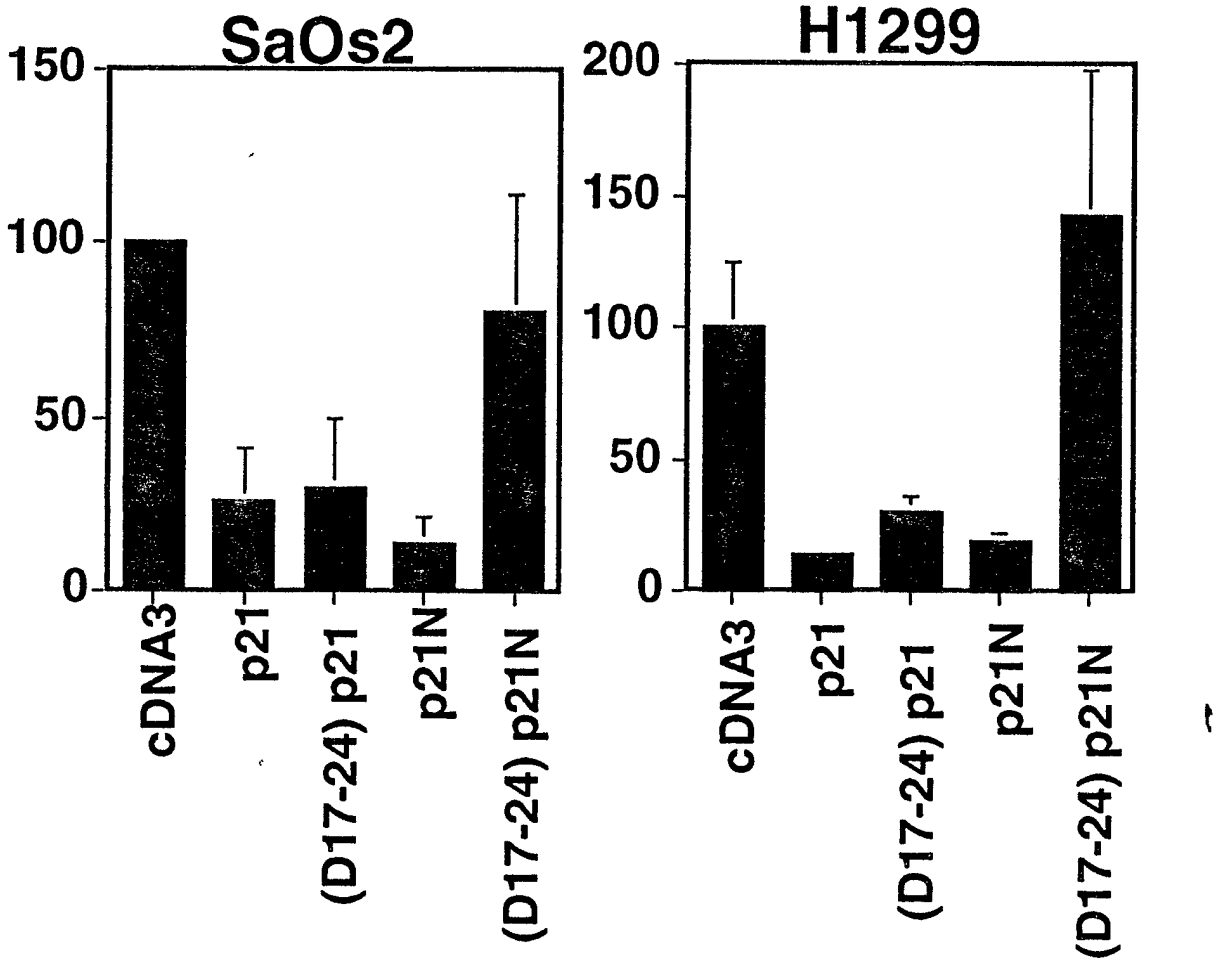
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
GSTp21Δ17-24
GSTp21
Protein (μM)
C
DNA replication (cpm incorporated)
20000
15000
10000
5000
0
None
PS160
PS161
peptide

Figure 9

A


SaOs2
H1299
150
100
50
0
200
150
100
50
0
cDNA3
p21
(D17-24) p21
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(D17-24) p21N
cDNA3
p21
(D17-24) p21
p21N
(D17-24) p21N

B

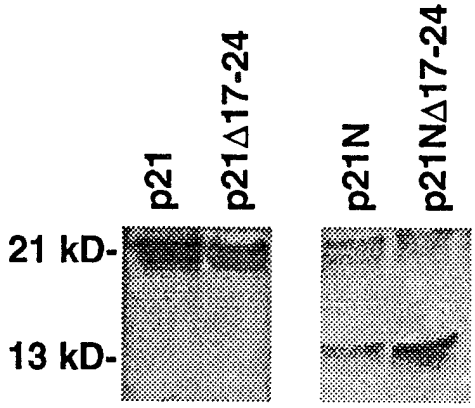
p21
p21Δ17-24
p21N
p21NΔ17-24
21 kD-
13 kD-

Table 1 p21 associates with several cyclin/cdk complexes through the cyclin-binding motif at amino acids 17-24 .

Protein	I.P. with	Association with p21					
		wt	D17-24	D53-58	D53-58 +PS100	D53-58 D53-58 +PS101	D17-24,
cdk2	a cdk2	+	+	-	n.d.	n.d.	-
cdk4	n.d.	-	n.d.	n.d.	n.d.	n.d.	n.d.
cyclin E	n.d.	+	-	+	n.d.	n.d.	n.d.
cyclin E-cdk2	a cdk2	+	+	+	-	+	-
cyclin A-cdk2	a cdk2	+	+	+	-	+	-
cyclin D1-cdk4	a cdk4	+	-	+	+	+	-

Two methods were used to generate this table. In the first method, radiolabeled cdks or cyclins were synthesized by *in vitro* transcription-translation in rabbit reticulocyte lysates (Fig. 5). Cyclins E, A and D1 were predominantly associated with the indicated rabbit cdks as evidence by immunoprecipitation with the relevant anti-cdk antibodies. A peptide containing the Cy2 site (PS102) gave results identical to PS100, while the mutant peptide (PS103) gave results identical to PS101.

In the second method, cyclin/cdk complexes were produced in insect cells by over-expression with a baculovirus expression system (see Table 2) and purified on glutathione agarose columns (Fig. 6). n.d.: not done.

Table 2 Derivatives of p21 with all or a subset of cyclin (Cy1 and Cy2) or cdk (K) binding sites were compared for their abilities to inhibit cyclin/cdk kinases. The Ki_{app} is presented for each inhibitor (nM).

	Binding sites on p21@			Cyclin E -cdk2	Cyclin A -cdk2	Cyclin D1 -cdk4
	Cy1	K	Cy2			
p21	+	+	+	1	0.1	8.5
p21D17-24	-	+	+	4.3	0.4	>850 *
p21D53-58	+	-	+	87	35	30
p21N	+	+	-	4	0.2	11
p21ND17-24	-	+	-	88	3.4	>1150*
p21C3D17-24	-	+	-	190	2.8	>952*
peptide PS100	+	-	-	296	220	>300000*
peptide PS102	-	-	+	32000	800	>300000*
peptide p21C2	-	-	+	6500	90	2000

Cyclin/cdk complexes were produced in insect cells by over-expression with recombinant baculoviruses and purified on glutathione agarose columns, kinase activity tested *in vitro* using bacterially produced GST-RbC as substrate. The phosphorylation of substrate was quantitated both by liquid scintillation counting of bands excised from gels and by phosphorimager analysis.

Ki_{app} is the concentration of inhibitor which inhibits kinase activity to 50% of activity seen in the absence of any inhibitor. @: Cy1, Cy2 and K: described in text. *>: inhibition to 50% basal activity is not achieved even at highest concentration of inhibitor tested (e.g. 850 nM). The synthetic peptides are described in the text. The p21 proteins were all produced and purified as GST-fusion proteins in bacteria. p21N (amino acids 1-90 of p21); p21C3 : deletion of amino acids 150-164 of p21(Cy2 site). D17-24: deletion of amino acids 17-24 (Cy1 site). D53-58: deletion of amino acids 53-58 (K site).

CONCLUSIONS

In the first year, we defined the domain of p53 that bound to RPA and constructed mutant p53 that failed to bind RPA, but still functioned as transcriptional activator. In the second year of this study, we examined whether these mutations of p53 affected other functions of p53, including transcriptional suppression and growth suppression activities. We found that while these mutations of p53 lost their ability to bind RPA, they could still function as transcriptional suppressor on the promoters lacking the p53 binding site and suppress cell growth in two tumor cell lines we tested. These results suggest that RPA-p53 interaction is not required for the growth suppression activity of p53. Instead, transcriptional activation and/or repression activity of p53 is responsible for the growth suppression activity of p53. These results were published this year (25). p53 has other functions relevant to cancer development and progression. p53 is required to induce apoptosis in response to irradiation or chemotherapy, to produce a pause in DNA replication after a sub-lethal dose of radiation so as to give the cell time to repair its DNA, and to prevent gene amplification. p53 can induce apoptosis independent of its transcriptional activation activity (26). p53 can also bind to insertion-deletion mismatch lesions (2) and function as an exonuclease (3). p53 may recruit RPA to these sites of DNA repair by p53-RPA interaction. Future experiments will be directed to test whether RPA-p53 interaction is important for these functions of p53.

The growth suppression activity of p53 is dependent on its ability to activate transcription. More and more evidences suggest that p21 is the major downstream effector of p53 in cell cycle control. p21 can be transcriptional up-regulated by p53 upon DNA damage. p21 can also suppress cell growth when overexpressed in vivo. In order to understand how p53, through regulating p21 level, controls the cell cycle upon DNA damage, we started to study how p21 directly inhibits the cell cycle machinery. In the first year, we separated domains of p21 responsible for interacting with and inhibiting cyclin-cdk and PCNA. We found that cdk kinases were the primary target for inhibition of double-stranded DNA replication in *Xenopus* extracts by p21 and for growth suppression in transformed cells while PCNA was the limiting target in the SV40 replication reaction (12). In the second year of this study, we further examined the importance of p21-PCNA interaction in vivo (27). By employing microinjection technique, we showed that the PCNA-interactiong domain or Peptide can inhibit cell growth in vivo. This is the first time that a PCNA-inhibitory domain of p21 has been shown to be important for its growth suppression activity (27).

In the first year, we found the cdk kinases were the primary targets of p21 in growth suppression assay. Here we report that p21 interacted with both cyclin and cdk subunits of the cyclin-cdk complexes. We also demonstrated that p21 used a highly conserved motif (we named it as cyclin-binding motif) to interact with cyclins. These results agree with the crystal structure of p27-cyclin A-cdk2 complex (17). This cyclin-binding motif is not only important for the kinase inhibitory activities of p21, but also important for the biological activities of p21. Cyclin-binding motifs are highly conserved among the family of p21, p27, p57. Sequences similar to the cyclin binding motif are also found in other cell cycle regulatory proteins, such as p107 (22) and E2F1 (28). Based on our studies of p21 and crystal structure of p27-cyclinA-cdk2 complex, we suggest that the conserved cyclin binding motifs in p57, p107 and E2F1 interact specifically with cyclins. In some cases (p107, E2F1) the Cy motif may be an accessory site to target the substrate to a cyclin/cdk kinase. Further experiments will be conducted to test whether the cyclin-binding site is important for facilitating the formation of p21/cyclin/cdk complexes in vivo and whether p21 can disrupt the association of other cell cycle regulatory proteins with cyclin/cdk complexes by utilizing the cyclin-binding motif.

REFERENCE

1. A. J. Levine, J. Momand, C. A. Finlay, *Nature* **351**, 453-456 (1991).
2. S. Lee, B. Elenbaas, A. Levine, J. Griffith, *cell* **81**, 1013-1020 (1995).
3. T. Mummenbrauer, et al., *cell* **85**, 1089-1099 (1996).
4. A. Dutta, J. M. Ruppert, J. C. Aster, E. Winchester, *Nature* **365**, 79-82 (1993).
5. W. S. el Deiry, et al., *Cell* **75**, 817-25 (1993).
6. C. Deng, P. Zhang, J. W. Harper, S. J. Elledge, P. Leder, *Cell* **82**, 675-84 (1995).
7. Y. Xiong, et al., *Nature* **366**, 701-4 (1993).
8. J. W. Harper, G. R. Adami, N. Wei, K. Keyomarsi, S. J. Elledge, *Cell* **75**, 805-16 (1993).
9. M. K. Shivji, S. J. Grey, U. P. Strausfeld, R. D. Wood, J. J. Blow, *Current Biology* **4**, 1062-8 (1994).
10. U. P. Strausfeld, et al., *Current Biology* **4**, 876-83 (1994).
11. S. Waga, G. J. Hannon, D. Beach, B. Stillman, *Nature* **369**, 574-8 (1994).
12. J. Chen, P. K. Jackson, M. W. Kirschner, A. Dutta, *Nature* **374**, 386-8 (1995).
13. K. Polyak, et al., *Cell* **78**, 59-66 (1994).
14. H. Toyoshima, T. Hunter, *Cell* **78**, 67-74 (1994).
15. S. Matsuoka, et al., *Genes and Development* **9**, 650-662 (1995).
16. M. H. Lee, I. Reynisdottir, J. Massague, *Genes & Development* **9**, 639-49 (1995).
17. A. A. Russo, P. D. Jeffrey, A. K. Patten, J. Massague, N. P. Pavletich, *nature* **382**, 325-331 (1996).
18. J. Koh, G. H. Enders, B. D. Dynlacht, E. Harlow, *Nature* **375**, 506-510 (1995).
19. P. K. Jackson, S. Chevalier, M. Philippe, M. W. Kirschner, *Journal of Cell Biology* **130**, 755-69 (1995).
20. M. Nakanishi, R. S. Robetorye, G. R. Adami, O. M. Pereirasmith, J. R. Smith, *Embo Journal* **14**, 555-563 (1995).
21. J. W. Harper, et al., *Molecular Biology of the Cell* **6**, 387-400 (1995).
22. L. Zhu, E. Harlow, B. D. Dynlacht, *Genes & Development* **9**, 1740-52 (1995).
23. R. Pepperkok, in *Cell Cycle: Materials and Methods* M. Pagano, Eds. (Springer-Verlag, Heidelberg, 1995) pp. in press.

24. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular cloning:a laboratory manual.* (Cold Spring Harbor Laboratory Press, Plainview, 1989), vol. 2.

25. L. M. Leiter, J. Chen, T. Marathe, M. Tanaka, A. Dutta, *Oncogene* **12**, 2661-2668 (1996).

26. C. Caelles, A. Helmberg, M. Karin, *Nature* **370**, 220-223 (1994).

27. J. Chen, et al., *Nucleic Acids Research* **24**, 1727-1733 (1996).

28. W. Krek, G. Xu, D. M. Livingston, *Cell* **83**, 1149-1158 (1995).